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High concentrations of myeloperoxidase in the equine uterus as an indicator of endometritis

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ABSTRACT

Intraluminal fluid and excessive abnormal hyperedema are regularly used for the diagnosis of endometritis in the mare, which is routinely confirmed by the presence of neutrophils on endometrial smears. Studies show a relation between neutrophils and myeloperoxidase (MPO), an enzyme contained in and released by neutrophils during degranulation or after cell lysis. This enzyme has been found in many fluids and tissues, and associated with different inflammatory pathologies in the horse. The aims of this study were to assess the presence and concentration of MPO in the equine uterus, and to investigate its relation with neutrophils, and other clinical signs of endometritis. Mares ($n = 51$) were evaluated for the presence of intraluminal fluid and excessive endometrial edema before breeding, and a small volume lavage and cytology samples were obtained. From 69 cycles, supernatant of the uterine flushes was analyzed with a specific equine MPO ELISA assay to measure MPO concentration. Cytology samples were used for the diagnosis of endometritis. Myeloperoxidase was present in the uterus of all estrus mares in highly variable concentrations. Myeloperoxidase concentrations were significantly ($P < 0.05$) higher in samples with positive cytologies and in the presence of intraluminal fluid. Occasionally, some samples with negative cytologies showed high MPO concentration, but the opposite was never observed. Cycles presenting hyperedema weren't associated with high concentration of MPO, intraluminal fluid, or positive cytology, making it a poor diagnostic tool of endometritis.

1. Introduction

Breeding mares results in a transient physiological inflammation of the endometrium [1–3]. This occurs as a response to semen, seminal plasma, extender, and/or bacteria present within the uterine lumen, but other factors including anatomical abnormalities and uterine degeneration can also contribute to its development [4]. Failure of this inflammation to resolve majorly affects the mare's fertility [1,5–8].

Activation of various inflammatory mediators increases vascular permeability, induces the influx of serum proteins and immunoglobulins (Ig) into the uterine lumen [9,10], and migration of polymorphonuclear neutrophils [9–12]. Clinical signs such as intraluminal fluid [4,13] and to a lesser extent, the presence of an abnormal quantity in edema of the endometrial folds, so called hyperedema [7,14], are used as practical tools to diagnose the pathology. The presence of inflammatory cells, mainly neutrophils within the endometrial lumen or wall can be used as a marker of endometritis [10,11,15,16]. Uterine cytology and biopsy are commonly performed to confirm a suspicion of the condition [17], whereas uterine culture may identify the eventual bacteria involved.

A better understanding of the mechanisms and processes involved in this pathology is necessary to improve the management of susceptible mares, and increase their pregnancy

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rate. Various inflammatory markers of the uterine environment have already been investigated [18–20]. In the horse, myeloperoxidase (MPO) has been found in many fluids and tissues, and associated with different inflammatory pathologies [21,22]. Myeloperoxidase is a prooxidant enzyme contained in and released by neutrophils during degranulation or after cell lysis [23,24]. In the field of equine reproduction, MPO has been mainly studied in sperm [25–28], but to our knowledge, presence of the enzyme and its significance in the uterus of the mare has not been investigated yet.

The objective of this study was to assess the presence of MPO in equine uterine lumen during estrus, to measure its concentrations, and to determine its relation with endometritis and its associated clinical signs.

2. Materials and methods

2.1. Mares and samples collection

The study was performed on mares presented for insemination with fresh or frozen semen, at Linalux-MLS Equine Reproduction Center between April 2011 and January 2013, without interfering with the normal monitoring and insemination procedure. All samples were obtained before breeding during a complete routine reproductive examination. A total of 51 Warmblood, Arabian, Lusitanian, Quarter Horse, and Draft Horse mares, aged from 5 to 23 years (mean: 13.4) were included, and 69 cycles were exploited. Pregnancy diagnosis was routinely performed at Day 14 with ultrasonography.

Mares were regularly scanned until a follicle greater than 35 mm was observed, when all samples and data were collected. Swabs and uterine lavages were obtained from all mares. After a vulvar scrub, a uterine cotton swab (Equivet; Kruuse, Marselv, Denmark) or a double-guarded uterine Cytology Brush (Minitüb, Tiefenbach, Germany) was passed per vagina into the uterus, and an endometrial cytology sample was obtained. Cytologic slides were air dried and stained with Diff-Quick (RAL, Martillac, France).

Using a sterile double-sleeve technique and a sterile insemination pipette (Minitüb), a low-volume (60 mL) uterine lavage was performed with Ringer's lactate. After a uterus massage the fluid was reaspirated into EDTA and/or dry tubes, and samples were centrifuged at 600× g for 10 minutes immediately. For technical reasons, some samples were collected in EDTA (n = 26) and some others (n = 38) in dry tubes. To validate the assay for both tubes, five samples were collected in EDTA and dry tubes. Supernatants were collected in 2 mL tubes, and stored at −20 °C until MPO analysis. Pellets were smeared on a glass slide, air dried and stained with Diff-Quick for cytologic examination.

2.2. Intraluminal fluid and edema grade

The quantity of intraluminal fluid was measured using ultrasonography. More than 1 cm of liquid was scored as abnormal for statistical analysis.

Edema was graded with a subjective scoring system slightly modified from a previous description [29]. Grade 0 was scored in the absence of edema; grade I when uterine folds were difficult to identify; grade II when some of the endometrial folds could be identified and the cervix had a fish-bone appearance; grade III when endometrial folds were easily identified with hyperechoic borders and hypoechoic centers (cartwheel); and grade IV for mares with "hyperedema", where endometrial folds were abnormally thick, and the normal architecture of the cartwheel was lost.

2.3. Cytology

A minimum of 10 fields were evaluated microscopically (400×) by the same examiner. The number of neutrophils per field was used to evaluate the degree of inflammation. For statistical analysis, an average of one or more neutrophil per field at 400× magnification was considered a sign of inflammation as previously described [7,30].

2.4. Myeloperoxidase analysis

Concentration of MPO was determined by a commercial ELISA (Bioptis SA, Liège, Belgium) developed by Frank, et al. [24] The primary antibody, rabbit IgG against MPO, was coated onto microplate wells (Cliniplate EB; Thermo Labsystems, Helsinki, Finland). Equine MPO standard (0, 1.015, 2.03, 4.06, 8.12, 16.25, 32.5, and 65 ng/mL) and low-volume uterine flush supernatants diluted 50× and 200× were added (100 μL) into the wells, and microplates were incubated overnight at 4 °C. After the plates were washed with 0.9% NaCl solution containing 0.1% Tween 20 (Sigma Chemical Company, St. Louis, MO, USA) the immobilized antibody–antigen complexes were incubated for 2 hours at 37 °C with the secondary antibody, guinea pig IgG against equine MPO labeled with alkaline phosphatase. After another washing, phosphatase activity was determined by incubation for 30 minutes at 37 °C in the dark with a paranitrophenyl phosphate stabilized solution. The reaction was stopped with 2.5 M NaOH and the absorbance at 405 nm read with the Multiscan Ascent Plate Reader (Thermo Labsystems). The absorbance was directly proportional to the activity of alkaline phosphatase bound to the secondary antibody against MPO and thus to the concentration of MPO immunocaptured from the sample. Each sample was assayed at two dilutions (50× and 200×), and when a concentration of MPO was obtained at both dilutions, the mean was calculated and taken into consideration. The mean value of coefficient of variation (CV) intraassays was 14.92 ± 13.15%, and 74% of the samples have CV lower than 14.92%. The CV greater than 20% corresponds to samples situated at the lower and upper limit of detection of the calibration curve. Despite a double dilution (50× and 200×) to obtain values for low and high concentration samples, some remained out of calibration curve, and they were marked as higher than 7000 ng/mL (i.e., the upper limit of detection).

2.5. Statistical analysis

For discontinuous data such as the presence of uterine liquid, hyperedema, pregnancy, and cytology, Fisher's test was used to determine the significance of contingency table. Normal distribution of parameters was tested with Kolmogorov–Smirnov test. As values were non-normally